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Enzymatic and Metabolic Transformation of Silybin and its Congeners

Mgr. Kateřina Purchartová

Supervisor: prof. Ing. Vladimír Křen, DrSc.

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Disertační práce

Enzymové a metabolické přeměny silybinu a vybraných flavonoidů

Mgr. Kateřina Purchartová

Školitel: prof. Ing. Vladimír Křen, DrSc.

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I hereby declare that this thesis is based on my own research carried out in the Laboratory of Biotransformation, Institute of Microbiology, Czech Academy of Sciences, except where due acknowledgement has been made in the text, and that all sources of information are cited. No part of the work was used for obtaining the same or different academic title.

In Prague Mgr. Kateřina Purchartová

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Abstract

Natural flavonoids and flavonolignans feature beneficial properties for living organisms such as antioxidant and hepatoprotective effects, anticancer, chemoprotective, dermatoprotective and hypocholesterolemic activities. Their metabolism in mammals is complex, the exact structure of their metabolites still remains partly unclear and the standards are usually not commercially available. Hence, this project focused on the preparation of potential and defined biotransformation Phase II sulfated metabolites of silymarin flavonolignans: silybin, 2,3-dehydrosilybin, isosilybin, silychristin, silydianin and flavonoids quercetin, taxifolin, rutin and isoquercitrin. Pure sulfated derivatives were prepared using aryl sulfotransferase from *Desulfitobacterium hafniense* and aryl sulfotransferase from rat liver.

Using heterologously expressed PAPS (3′-phosphoadenosine-5′-phosophosulfate) - independent arylsulfotransferase from *Desulfitobacterium hafniense* and cheap *p*-nitrophenyl sulfate as sulfate donor, sulfated flavonolignans and flavonoids were obtained in high yields. Silymarin flavonolignans afforded exclusively monosulfates at the position C-20 (C-19 in the case of silychristin), except 2,3-dehydrosilybin that yielded also the 7,20-*O*-disulfated derivative. Isoquercitrin and rutin were selectively sulfated at C-4′ position of the catechol moiety. Taxifolin was sulfated at the C-4′ position as well, however, a minor amount of the C-3′ isomer was also formed. Sulfation of quercetin proceeded preferentially at the C-3′ position, but a lower proportion of the C-4′ isomer was isolated as well.

On the contrary, recombinant mammalian PAPS-dependent aryl sulfotransferase was less efficient and had a narrower substrate specificity. The enzyme from rat liver catalyzed only sulfation of silybin B (at C-20), quercetin and taxifolin (at C-3′) as evidenced from isolated products. Silybin A and the quercetin glycosides (rutin and isoquercitrin) remained intact.

The sulfated products prepared by both aryl sulfotransferases were fully characterized by HRMS and NMR methods. The sulfated metabolites can be used for *in vitro* evaluation of biological activities and as authentic standards for metabolic studies *in vivo*.

Abstrakt

Flavonolignany jsou přírodní látky, které mají mj. chemoprotektivní, hepatoprotektivní, antioxidační, dermatoprotektivní účinky. Tyto přírodní látky se metabolizují zejména ve II. fázi biotransformace, přičemž jednou z dominantních metabolických drah je sulfatace. Detailní struktura většiny takto vzniklých metabolitů však není dosud známa a jejich standardy nejsou komerčně dostupné. Cílem této studie bylo připravit sulfatované deriváty flavonolignanů silymarinu - silybinu, 2,3-dehydrosilybinu, isosilybinu, silychristinu a silydianinu a různých flavonoidů - kvercetinu, isokvercitrinu, taxifolinu a rutinu, a tyto potenciální savčí metabolity plně charakterizovat. Pro tento účel byly použity dvě rekombinantní aryl sulfotransferasy: 1. z *Desulfitobacterium hafniense*, 2. z potkaních jater. Sulfáty byly získány ve vysokých výtěžcích za použití *p*-nitrofenyl sulfátu jako donoru SO_3^- skupiny a PAPS (3'-fosfoadenosin-5'-fosfosulfát)-independentní bakteriální aryl sulfotransferasy z *D. hafniense*. Flavonolignany tvořily zcela výlučně monosulfatované deriváty, kde pozice sulfátu byla určena na pozici C-20 (nebo C-19 v případě silychristinu). Pouze 2,3-dehydrosilybin podléhal další sulfataci a bylo možné isolovat také jeho 7,20-*O*-disulfatovaný derivát. Isoquercitrin a rutin byly selektivně sulfatované v poloze C-4' katecholové skupiny. Také taxifolin byl preferenčně sulfatován v této poloze (C-4'), ale bylo také zjištěno menší množství C-3´isomeru. Naopak sulfatace kvercetinu probíhala přednostně na C-3' poloze, ale opět byla prokázána přítomnost C-4' isomeru.

Naopak rekombinantní savčí aryl sulfotransferasa, která je PAPS-dependentním enzymem, byla méně katalyticky účinná s užší substrátovou specificitou; byly izolovány pouze sulfáty silybinu B (C-20), kvercetinu a taxifolinu (C-3´). Bylo prokázáno, že silybin A a glykosidy kvercetinu (rutin a isokvercitrin) nejsou tímto enzymem přeměňovány.

Sulfatované produkty studovaných látek připravené pomocí obou aryl sulfotransferas byly plně charakterizovány pomocí HRMS a NMR. Tyto sulfatované metabolity mohou být následně použity pro *in vitro* studie biologické aktivity a dále jako autentické standardy při metabolických studiích *in vivo*.

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List of Abbreviations

AST	Aryl Sulfotransferases
AST DH	Aryl Sulfotransferase from *Desulfitobacterium hafniense*
AST IV	Aryl Sulfotransferases from Rat Liver
COMT	Catechol *O*-Methyl Transferases
COSY	Correlation Spectroscopy
CYP	Cytochrome P450
DMSO	Dimethyl Sulfoxide
GST	Glutathione *S*-Transferases
HPLC	High Performance Liquid Chromatography
HSCCC	High-Speed Counter-Current Chromatography
HSQC	Heteronuclear Single Quantum Coherence
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
LB	Lysogeny broth
LC-MS	Liquid Chromatography Mass Spectrometry
MS	Mass Spectrometry
NBD	Nucleotide Binding Domain
N.D.	Not Detected
NMR	Nuclear Magnetic Resonation
PAP	3′-Phosphoadenosine 5′-Phosphate
PAPS	3′-Phosphoadenosine-5′-Phosphosulfate
p-NP	*p*-Nitrophenol
p-NPS	*p*-Nitrophenyl Sulfate
SDS–PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SPE	Solid Phase Extraction
SULT	Sulfotransferase
TLC	Thin Layer Chromatography
TB	Terrific Broth
UDP	Uridine Diphosphate
UDP-GA	Uridine Diphospho-Glucuronic Acid
UGT	UDP - Glucuronyltransferase

Experiments not performed by the author

Experiment:	**Performed by:**
Cloning	Leonie Engels (RWTH, Aachen, DE)
Preparative HPLC experiments	David Biedermann (IMIC CAS)
MS analysis	Miroslav Šulc (IMIC CAS)
HRMS/ MS analysis	Josef Cvačka (IOCB CAS)
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Kinetics of regioisomeric taxifolin and quercetin sulfate formation	Alena Křenková (IMIC CAS) Kateřina Valentová (IMIC CAS)

1 Introduction

Flavonoids are naturally occurring polyphenols, which play a prominent role in the human diet as food supplements or herbal medicines. They are valued as abundant micronutrients widely distributed in fruits, vegetables, and beverages such as wine, cocoa and tea[1]. Flavonoids are divided into six major subclasses, according to their structures: e.g. flavones, flavonols, isoflavons, flavanons, flavanols (catechins) and anthocyanidins. Flavonolignans are specific derivatives of flavonoids, which resulted from the condensation of a flavonoid (taxifolin, quercetin, luteolin and some others) and coniferyl alcohol or its derivatives (lignan moiety)[2].

Various epidemiological studies demonstrated that a flavonoid-rich diet could lower the risk of a range of degenerative diseases, like cardiovascular disease, diabetes or cancer[3,4]. Flavonoids are generally recognized as potent antioxidants and radical scavengers but they have also further biological activities as anti-allergic, anti-inflammatory, anti-microbial and anti-cancer properties. Therefore, flavonoids are employed for disease prevention and health improvement[5,6]. Some flavonoids naturally occur as aglycones, however more often the compounds can be found as conjugates with sugars (glycosides)[7].

In the last decades, since the flavonoids became popular as food supplements, their daily intake has increased up to hundreds of mg per day[8]. Pharmacokinetics, pharmacodynamics, and pharmacological mechanisms of action and metabolism of flavonoids were studied extensively. Generally, the metabolism of flavonoids starts in the stomach and in small intestine and the post-absorption modifications occur in liver and in other organs[9]. The absorption depends mainly on the structure of the xenobiotic. E.g. quercetin glucosides are absorbed more rapidly than their aglycone[10-12] or other quercetin glycosides (rutin)[13-15]. Moreover, some flavonoids and their derivatives (especially glycosides) can pass up to the *caecum*, where they are cleaved and metabolized by intestinal microflora. The enzymatic systems of glycosidases (*i.e.* α-arabinofuranosidase, L-α-rhamnosidase, α-fucosidase, and β-glucosidase) and other bacterial enzymes degrade flavonoids into small molecules – mainly phenolic acids[1,7].

The resorbed flavonoid molecules are biotransformed mainly by the Phase II conjugation reactions rather than metabolized by Phase I reactions, e.g. by cytochromes P450. It was found that the biotransformation of flavonoids and other polyphenols preferably follow the pathways *via* sulfation, glucuronidation or methylation by sulfotransferases (SULT), UDP-glucuronyltransferases (UGTs), *N*-acetyltransferases,

glutathione *S*-transferases, thiopurine-*S*-methyltransferases and catechol *O*-methyl transferases (COMT)[16].

The present study focuses on silymarin flavonolignans and flavonoids, namely on quercetin, taxifolin, quercetin-3-*O*-β-D-glucopyranoside (isoquercitrin), and quercetin-3-*O*-rutinoside (rutin, *Figure* ***1.1***). Preparation of sulfated metabolites of these compounds by various sulfotransferases, related structural problems and basic biological properties will be described and discussed in detail.

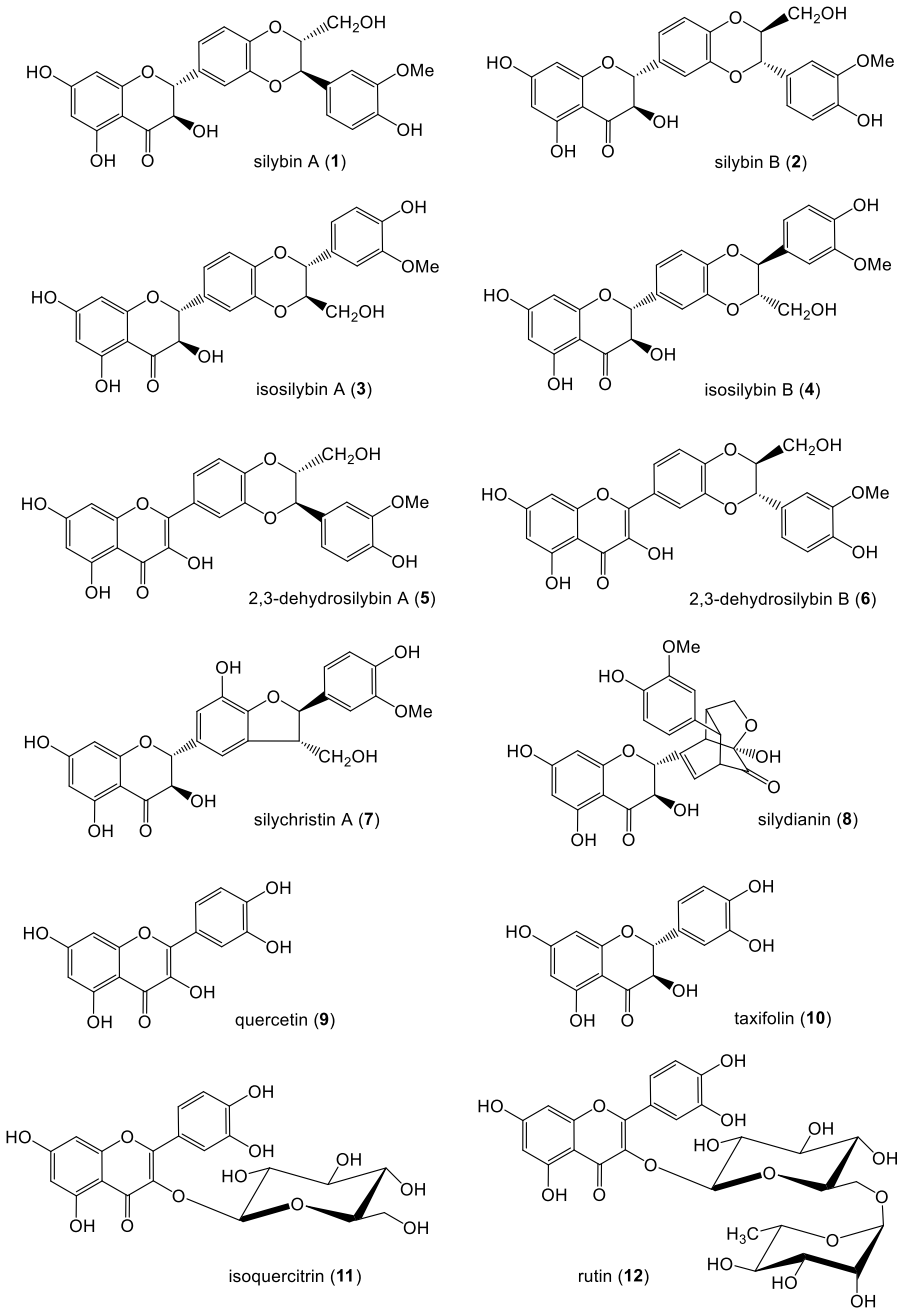

Figure 1.1*: Structures of the (parent) compounds studied.*

2 Silymarin Components

Silymarin is a crude extract from milk thistle (*Silybum marianum*, (L.) Gaertn., Asteraceae) fruits, which has been in human nutrition since ancient times[17]. Silymarin complex is composed of flavonolignans (such as silybin, 2,3-dehydrosilybin, isosilybin, silychristin, silydianin) and a flavonoid taxifolin. Silymarin flavonolignans are biogenetically formed by the oxidative radical coupling of coniferyl alcohol to the catechol moiety of taxifolin. This non-selective reaction generates a vast variety of their congeners. Most of them occur in nature as two diastereoisomers in various proportions: silybin A and B, 2,3-dehydrosilybin A and B, isosilybin A and B, and silychristin A and B; only silydianin exists as a single isomer[18]. Silymarin flavonolignans possess strong antioxidant and hepatoprotective effects. Furthermore the compounds exhibited anti-cancer, chemoprotective, dermatoprotective and hypocholesterolemic activities[19]. The flavonolignans are barely soluble in water, the plasma concentrations after oral administration in humans were approx. in the scale of ng/ml[20,21]. Therefore, *in vivo* activity of flavonolignans is mainly influenced by their bioavailability. Solubility and bioavailability of the compounds can be enhanced by the use of advanced drug delivery systems such as liposomes, phytosomes, self-emulsifying drug delivery systems, solid dispersion systems, nanosuspensions, floating tablets or by micronization[22] (Appendix 6). Metabolism, pharmacokinetics and possible drug-drug interactions of the flavonolignans represent another important topic. Despite numerous attempts, the exact structures of silymarin metabolites remain unexplored. Except for silybin, no detailed studies exist concerning metabolic processing of each flavonolignan. The most relevant information on the biotransformation of silybin and some other congeners were recently reviewed by us[16] (Appendix 7). The data for each particular compound used in this study will be discussed separately.

2.1 Silybin

2.1.1 General Information

Silymarin contains between 30 to 50 % of silybin (also referred to as silibinin). Natural silybin is a quasi-equimolar mixture of silybin A (**1**) and silybin B (**2**, *Fig.* ***1.1***). Optically pure silybin diastereisomers can be prepared by chromatographic separation of their glycosides[23]. The separation of **1** and **2** can be also achieved by preparative HPLC[24] or by chromatographic separation of their peracetylated galactosides[25]. The most efficient method

is based on selective alcoholysis of silybin 23-*O*-acetates with Novozym 435[26,27]. *In vivo*, silybin is widely used as a hepatoprotectant[19], but *in vitro* its antioxidant, anti-radical, anti-cancerogenic[28], chemoprotective and hypocholesterolemic activities[29] were reported as well. It supported redox homeostasis in several *in vitro* and *in vivo* models[30], displayed cytoprotective effects[30] and interacted with specific receptors[19]. Silybin biological activity is strongly dependent on its chirality: silybin B and silybin A displayed differences in their biological properties[31,32].

2.1.2 Metabolism of Silybin

Interaction with Phase I Biotransformation Enzymes

Studies published in last decades showed that silybin is modified by cytochromes P450 (CYPs). *In vitro* silybin inhibits CYP1A1[33], CYP2C9[34], CYP2D6[35], CYP3A4[34]. On the other hand, only negligible effect of silybin on various CYPs was reported *in vivo*, which could be explained mainly by its low systemic concentration[16]. Only single silybin metabolite formed probably by CYP2C8, demethylsilybin (*nor*-silybin), was confirmed by MS and LC-MS[36]. A recent *in vivo* study in rats revealed traces of methylated derivatives of silybin B. The only identified methylated metabolite was 20-*O*-methyl silybin B[37].

Interaction with Phase II Biotransformation Enzymes

Silybin, like other polyphenolic substances, undergoes quick conjugation reactions. Most of the published metabolic studies were performed on natural silybin or crude silymarin. Some authors tried to analyze silybin diastereoisomers metabolites separately, however, the absolute configurations of respective metabolites were not determined[38,39]. Only two studies elucidated silybin metabolism in more details[37,40]. The conjugation was strictly stereoselective; silybin A and silybin B had different conjugation profiles[37-41]. Silybin B was rather glucuronidated[37], whereas silybin A was sulfated and glucuronidated in about the same ratio[40], however at a substantially lower extent than silybin B[37]. A plethora of different silybin B glucuronides and sulfates were detected (by MS methods), but only silybin B 7-*O*-β-D-glucuronide (major plasma metabolite) and silybin B-20-*O*-sulfate were identified by comparison with authentic standard. Complex metabolites (diglucuronides, glucuronidated and sulfated metabolites) of silybin B were detected as well, but the position of glucuronidation and/or sulfation remained unknown[37].

Chemical or *in vitro* Preparation of Potential Silybin Metabolites

Silybin A&B 23-*O*-sulfate [42] and 7,23-disulfate of optically pure silybin A and B[43] were synthesized chemically, while silybin A&B 7-*O*-sulfate was prepared by using the fungus *Cunninghamella blakesleana*[44]. There exist four papers dealing with silybin glucuronidation[41,45-47], where the metabolites were fully characterized (^{1}H, ^{13}C NMR and MS). The 7-*O*-β-D-glucuronide, 20-*O*-β-D-glucuronide, and 5-*O*-β-D-glucuronide of optically pure silybin B were prepared by using ovine liver microsomes[41]. Silybin 20-β-D-glucuronide was established to be a major silybin B conjugate in humans, while the C-7 regioisomer was also formed, but in a lower proportion[41]. Han *et al.* confirmed this statement, moreover characterized missing 7-*O*-β-D-glucuronide and 20-*O*-β-D-glucuronide of silybin A, which were prepared with bovine liver microsomes[46]. These compounds can also be prepared at the preparatory scale by *Streptomyces* sp. strain without using expensive uridine diphospho-glucuronic acid (UDP-GA)[47] or by using bovine liver microsomes with UDP-GA[45]. Silybin 7-*O*-β-D-glucuronide was shown to be a better antioxidant than non-conjugated silybin[41]. The sulfation at C-23 position of silybin did not influence its antiradical activity[42], whereas sulfation at C-7 reduced it[44]. Silybin A&B 7,23-*O*-disulfate was a slightly worse inhibitor of bladder cancer HTB9 and colon cancer HCT16 cells growth, but a better growth inhibitor of prostate carcinoma PC3 cells compared to non-conjugated silybin[43].

2.1.3 Bioavailability of Silybin

Silybin suffers from low aqueous solubility (ca 0.5 g/l, physiological conditions), which strongly depends on pH (increases at alkaline pH). When silybin is administered *p.o.* in high doses (up to 7 g/day) it is not well absorbed. The oral bioavailability was estimated to be 0.3 % for silybin B and 0.03 % for silybin A[37]. There are plenty of attempts aiming at improving silybin solubility and bioavailability[22] (Appendix 6).

2.1.4 Pharmacokinetics of Silybin

Comparison of pharmacokinetic studies, performed with optically pure silybin diastereomers (administered separately) and natural silybin (A&B), revealed strong mutual influence of both diastereoisomers on their particular metabolic transformations[37]. Compounds **1** and **2** were both absorbed and eliminated rapidly, but they displayed different pharmacokinetic profiles. Silybin A was conjugated slower and to a lower extent than silybin B. 12 % of total silybin A remained unconjugated (compared to 3 % of silybin B)[37,48] and reached T_{max} at about 4 h (2.6 h for silybin B)[37]. Moreover, reported C_{max} for free silybin B was 3.5 times higher than that one for unconjugated silybin A[37,49,50]. Silybin in mice was

quickly absorbed after oral application, distributed to bile and 30 minutes after administration, silybin conjugates (not specified) but also unconjugated silybin were detected in various organs[51].

2.2 Isosilybin

2.2.1 General Information

Isosilybin (also referred to as isosilibinin) is another flavonolignan present in the seeds of milk thistle[19,52]. In silymarin, isosilybin occurs as isosilybin A (**3**) and isosilybin B (**4**, *Fig.* ***1.1***) in a ratio of about 4:1. Low isosilybin content in silymarin (< 10 %)[53] together with its extremely complicated isolation and purification contributes to its low availability (only tens of milligrams). Isosilybin diastereomers can be separated by HPLC, semipreparative HPLC or preparative HPLC[24,54]. Recently, preparative production of both optically pure isosilybin A and B in a purity over d.e. 95 % was published[55]. Isosilybin displayed superior efficacy over other silymarin constituents[28]. Similarly to silybin diastereoisomers, also isosilybin diastereoisomers differ substantially in their biological properties[32,56,57].

2.2.2 Bioavailability, Pharmacokinetics and Metabolism of Isosilybin

There is a lack of studies dealing with isosilybin pharmacokinetics or metabolism. There exists a single report on isosilybin sulfated and glucuronidated conjugates, which were prepared in perfused rat liver. Although the structures of these metabolites are unknown[40], sulfation was found to be the major metabolic pathway for both isosilybin diastereomers (**3**, 84 %; **4** 85 %). The concentration of 0.6 μg/ml for **3** and 0.1 μg/ml for **4** conjugates at 90 minutes corresponded to 28 % or 10 % of **3** or **4** dose, respectively[40].

2.3 2,3-Dehydrosilybin

2.3.1 General Information

2,3-Dehydrosilybin A and B (**5**, **6**, *Fig.* ***1.1)***, oxidized forms of silybin, are another flavonolignans, which occur as a minor components in silymarin[58]. 2,3-Dehydrosilybin can be prepared from silybin by dehydrogenation with iodine in acetate buffer or by heating in pyridine in the presence of oxygen[59]. 2,3-Dehydrosilybin is a potent radical scavenger and antioxidant, ca 3× better than silybin[59-61]. Its anti-cancer activity[62], inhibitory effect on P-glycoprotein, and cytotoxicity (both to cancer and to normal cells)[63] were reported as well.

2.3.2 Bioavailability, Pharmacokinetics and Metabolism of 2,3-Dehydrosilybin

There exist no data concerning bioavailability, pharmacokinetics or metabolism of 2,3-dehydrosilybin, probably due to its minor occurrence in silymarin.

2.4 Silychristin

2.4.1 General Information

Until recently, silychristin has been isolated in small quantities only. The quantity of silychristin in milk thistle fruits, depends on the site of origin and also the plant cultivar. Silychristin was initially considered to be a single isostereoisomer, but later studies reported silychristin A[64] and silychristin B[65] (ca 9 : 1, **7**, *Fig.* ***1.1***). In 2010, a preparative high-speed counter-current chromatography (HSCCC) affording hundreds of milligrams of pure silychristin (99.3 %) from silymarin was published[66]. In 2014, a novel preparatory method for the separation of the minor silymarin components using Sephadex LH-20 gel chromatography was published by our group[67]. Silychristin has often been discarded after the separation of silybin from silymarin, and, as a consequence, its chemistry and biological activities have remained mostly unexplored[68]. There are only a few papers dealing with pure silychristin[66-72]. Silychristin has more beneficial properties than other silymarin flavonolignans[68]. Its antioxidant and antiradical activity is twice as high as that of silybin[71,72].

2.4.2 Pharmacokinetics and Metabolism of Silychristin

There is only a single report on silychristin sulfated and glucuronidated conjugates, which were prepared in perfused rat liver[40]. Silychristin was sulfated and glucuronidated approximately at the same ratio and these metabolites were excreted into the bile[40]. The concentration of silychristin conjugates (in the perfusate) was 0.3 μg/ml at 90 minutes and corresponded to 5 % of the dose[40]. No structures (regioisomers) of the metabolites were determined so far.

2.5 Silydianin

2.5.1 General Information

Silydianin (**8**) belongs among the minor silymarin components (ca 5 – 10 %)[73,74]. This flavonoid was also identified in the leaves of *Cirsium japonicum*, a perennial herb found in Asia[75]. Silydianin significantly differs from other silymarin flavonolignans – it has a rather unique structure containing the hemiacetal moiety (*Fig.* ***1.1***). Until recently, silydianin could

be isolated only in small quantities using laborious procedures, e.g. preparatory HSCCC, and thus it was available only as an analytical standard[66]. Now, silydianin can be isolated from silymarin in high quantities and purity by Sephadex LH-20 gel chromatography[67]. There exist only a handful of the papers dealing with pure silydianin (or as a part of silymarin mixture or contained in *Cirsium japonicum*)[69,71,75]. Silydianin is a weaker antioxidant than silymarin complex[72], but it regulated caspase-3 activity, and thus it might have anti-inflammatory activity[76]. Silydianin displayed anti-adipogenic activity and inhibited the accumulation of lipids in adipocytes[75].

2.5.2 Pharmacokinetics and Metabolism of Silydianin

Silydianin is almost exclusively excreted by bile as a glucuronide (80 %)[40,50], but the exact structures (regioisomers) of the conjugated metabolites remain unknown[40,50]. When silydianin was administrated *p.o.* (single dose, standardized milk thistle extract, human) no silydianin sulfates or unconjugated silydianin were detected[50], whereas *in vitro* study revealed traces of silydianin sulfates[40]. After 50 minutes the concentration of silydianin conjugates (in rat liver perfusate) reached a plateau. The concentration of 0.1 μg/ml (at 90 minutes) corresponded to 4 % of the initial dose[40]. Silydianin pharmacokinetics and metabolite structures therefore remain a largely unexplored area.

3 Flavonols and Flavanonols

Flavonols are a major class of flavonoids in the human diet, among them quercetin[15], its glycosides rutin[77] and isoquercitrin[78] are largely used in food supplements and therefore they are in the focus of investigators. Taxifolin (flavanonol) is also used in nutraceuticals, although not as frequently as quercetin[79]. These flavonoids play a prominent role in the human diet as they are generally considered to be potent antioxidants, radical scavengers and chemoprotectants. Beside this, they have a plethora of other biological activities such as anti-allergic, anti-inflammatory, antimicrobial or anti-cancer properties. For these reasons they are now largely used for disease prevention and health improvement[6,12].

3.1 Quercetin

3.1.1 General Information

Quercetin (**9**, *Fig.* ***1.1***) naturally occurs as aglycone, but it is often conjugated with sugars (glucose, galactose, rhamnose or rutinose) as e.g. rutin, isoquercitrin, quercitrin etc.[7]. Quercetin possesses antioxidant, anti-fibrotic, anti-coagulative, anti-atherogenic, anti-hypertensive, anti-proliferative and anti-angiogenic activities[15].

3.1.2 Metabolism of Quercetin

Orally administered quercetin is mostly metabolized in the gastrointestinal tract (stomach, small intestine) prior to systemic uptake[80]. Passive diffusion and organic anion transporting polypeptide are involved in the intestinal absorption, but the mechanism of gastric absorption remains unknown. Absorption of quercetin and its conjugates from the gastrointestinal tract was highly variable (20 to 60 % in rats and 24 to 53 % in humans). Nevertheless, ingestion of quercetin or quercetin-rich foods is associated with significant increases in plasma free (traces) and conjugated quercetin levels in both rats and humans. No clear evidence of quercetin metabolism in Phase I was reported so far[80]. Quercetin is converted by UGTs, SULTs and COMTs to conjugated derivatives such as quercetin monoglucuronide, quercetin diglucuronide, quercetin sulfate, quercetin monoglucuronide sulfate and methylated quercetin monoglucuronide sulfate[80-82]. Other metabolites include methylated derivatives isorhamnetin and tamarixetin. Quercetin 3-*O*-β-D-glucuronide, quercetin 3′-methyl-3-*O*-β-D-glucuronide and quercetin 3′-*O*-sulfate were found to be major metabolites in humans[83,84]. Quercetin metabolites are then transported into the liver and/or kidney where they undergo further conjugation reactions[80] and finally quercetin is eliminated

via feces, bile or urine. Unabsorbed quercetin passes into the colon where it is de-conjugated and degraded by the microflora[85,86]. 3-Hydroxyphenylacetic acid, benzoic acid and hippuric acid were identified as major metabolites of quercetin[80]. In healthy human subjects, C4-radiolabeled quercetin *p.o.* was excreted *via* urine (3.3 %) or faeces (4.6 %) but the majority of the label was recovered as CO_2[87].

3.2 (+)-Taxifolin

3.2.1 General Information

(+)-Taxifolin (**10**, *Fig.* ***1.1***), also known as dihydroquercetin, is commonly isolated from the bark of various conifers, but it also occurs in milk thistle, fruits (grapes and grapefruit[88]) or onions[89]. It is rarely used as a single compound, usually it occurs in complex preparations with other compounds such as ascorbic acid or milk thistle flavonoids. Taxifolin maintains normal function of the circulatory system[90], improves immune action, has pro-apoptotic activity[79] and inhibitory effect on lipid peroxidation[79]. Plethora of mechanisms identified in *in vitro* models[79] has mostly not been verified *in vivo*.

3.2.2 Bioavailability, Pharmacokinetics and Metabolism of Taxifolin

Trace amounts of taxifolin can be detected in plasma in rats after a single oral dose[91]. Due to the low solubility in water (1 mg/ml at 25 °C), poor absorption and subsequent metabolization, bioavailability and efficacy of taxifolin is low[92]. 3,4-Dihydroxyphenylacetic, *m*-dihydroxyphenylacetic and 3-methoxy-4-hydroxyphenylacetic acids were found to be major metabolites after *p.o.* administration in humans[93].

3.3 Isoquercitrin

3.3.1 General Information

Isoquercitrin (quercetin-3-*O*-β-D-glucopyranoside, **11,** *Fig.* ***1.1***), one of the main glycosidic derivatives of quercetin, can be found in numerous fruits, vegetables, cereals, nuts, wine or tea[78]. Isoquercitrin is not easy to isolate from natural materials. Recently an enzymatic method employing α-L-rhamnosidase (from *A. terreus* heterogeneously expressed in *P. pastoris*) was developed by our group to produce pure isoquercitrin (>99.5 %) from rutin in high quantities[94]. Isoquercitrin was reported to be a very efficient chemoprotective agent (both *in vivo* and *in vitro*); its antioxidant and antiradical activity

results in anti-cancer, anti-allergic, anti-diabetic effects and prevents human body against oxidative stress and related diseases[78].

3.3.2 Bioavailability, Pharmacokinetics and Metabolism of Isoquercitrin

Only few studies were performed on pure isoquercitrin, these compared its metabolism with that of quercetin and rutin[78]. Quercetin glucosides were absorbed more rapidly than other types of quercetin glycosides, such as rutin[13-15,95]. The bioavailability of isoquercitrin can be enhanced when administered together with high-fat diet, alcohol or non-digestible oligosaccharides[78]. Isoquercitrin is absorbed intact and then it is quickly eliminated and/or biotransformed to quercetin and isorhamnetin conjugates[96]. Moreover, isoquercitrin is hydrolyzed by intestinal bacteria (e.g. *Enterococcus casseliflavus*) and the aglycone can be further degraded to 3,4-dihydroxyphenylacetic acid and phloroglucinol, which is further metabolized by other gut bacteria[97,98]. Latest studies in this field discovered that quercetin, acetylated or dehydroxylated isoquercitrin and hydroxylated or hydroxymethylated quercetin are the major metabolites produced by human bacteria isolated from faeces[99]. An overview of all possible isoquercitrin metabolites is given in a recent review[78], however, their exact structures remain unclear due to the methods used (only HPLC, LC-MS).

3.4 Rutin

3.4.1 General Information

Rutin (quercetin-3-rutinoside, **12**, *Fig.* ***1.1***), is known also as a vitamin P. Together with its aglycone, quercetin it can be found in a plethora of edible plants like onions, apples, berries, tea and wine[100]. Rutin is abundantly distributed in fruits, and citrus and buckwheat seeds. Arial part of buckwheat (*Fagopyrum esculentrum* Moench, Polygonaceae) together with the Brazil tree Fava d´Anta (*Dimorphandra mollis* Benth., Fabaceae) are the main source of rutin[94,100]. Rutin has plenty of beneficial properties such as vascular endothelium protection, anti-cancer, anti-inflammation, anti-asthma, anti-microbial, antioxidant and antiradical properties[77,101,102]. Rutin displayed vasorelaxant and hypotensive effect[103] and it is commonly used in various formulations.

3.4.2 Bioavailability and Pharmacokinetics of Rutin

Rutin is poorly soluble in water[104] and thus its bioavailability is rather low (ca 20 %). Rutin cannot be absorbed in its native form and it is mainly hydrolyzed to quercetin in large

intestine by caecal microflora[9]. Due to its multiple beneficial properties the acceptable dosage of rutin is up to 1 g/day[105].

3.4.3 Metabolism of Rutin

Even though rutin intake from edible plants and/or from food supplements is high, there is still a lack of information on exact rutin metabolism. Generally, rutin is considered to be mainly hydrolyzed by gut microflora and then absorbed (as quercetin)[106]. Some studies showed that rutin could be absorbed in the small intestine and in the large bowel, but slower and in a smaller extend than quercetin[107]. Little or no rutin was found to be absorbed, because of its complete metabolism by the gut microflora[102]. Quercetin, isoquercitrin, leucocyanidin and further degradation products like 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxytoluen, 3-hydroxyphenylacetic acid and 4-hydroxy-3-phenylacetic acid were found to be rutin metabolites[102]. Further metabolites like rutin sulfate, rutin glucuronide and methyl rutin were isolated from filamentous fungi. Unfortunately, authors only guess these metabolites by comparing their HPLC profiles with literature, where they were characterized only by LC-MS and ^{1}H-NMR[108].

4 Sulfotransferases

Sulfation, together with other conjugation reactions of xenobiotics in mammals, leads to polyphenols excretion from their bodies. This chapter will give a brief overview of 3′-phosphoadenosin-5′-phosphosulfate (PAPS) dependent and independent aryl sulfotransferases.

4.1 Eukaryotic Sulfotransferases

Sulfotransferases convert xenobiotics and eubiotics into metabolites that are more water-soluble and usually less toxic; this process mostly facilitates their excretion into bile or urine[109]. Sulfotransferases are present in various tissues including liver, kidney, brain, adrenals, gut and platelets[109]. The presence of a co-substrate, e.g. sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) is required for effective sulfate conjugation in eukaryotes. There are two groups of sulfotransferases: cytosolic and membrane-associated[110]. The cytosolic sulfotransferase superfamily consists of minimally ten functional (human) genes encoding enzymes catalyzing the sulfation of xenobiotics[111,112]. Membrane-associated sulfotransferases play a crucial role in various biological processes including viral entry into cells, leukocyte adhesion or anti-coagulation. Moreover sulfotransferases are responsible for the sulfation of various biomolecules, such as carbohydrates and proteins[113].

Aryl Sulfotransferases from Rat Liver (AST IV)

Aryl sulfotransferases (EC 2.8.2.1) catalyze the sulfation of a wide variety of phenolic-containing compounds[114]. Three isozymes (I, II, IV) were identified. Rat hepatic cytosolic isozyme IV is a major sulfotransferase and displays the broadest substrate specificity of the three isozymes[115]. Its substrates include a wide variety of phenols, some aromatic hydroxylamines and benzylic alcohols. Isozyme IV is substantially different from isozymes I and II, particularly in its catalytic flexibility[110,114]. AST IV can catalyze an 'exchange' reaction involving the PAP-dependent transfer of sulfate from a donor (e.g. *p*-nitrophenyl sulfate, *p*-NPS) to an acceptor substrate *(Fig.* ***4.1****)*[116]. Sulfation catalyzed by this isozyme is influenced by substrate charge distribution factors[116], hydrophobicity and stereochemistry[110,114].

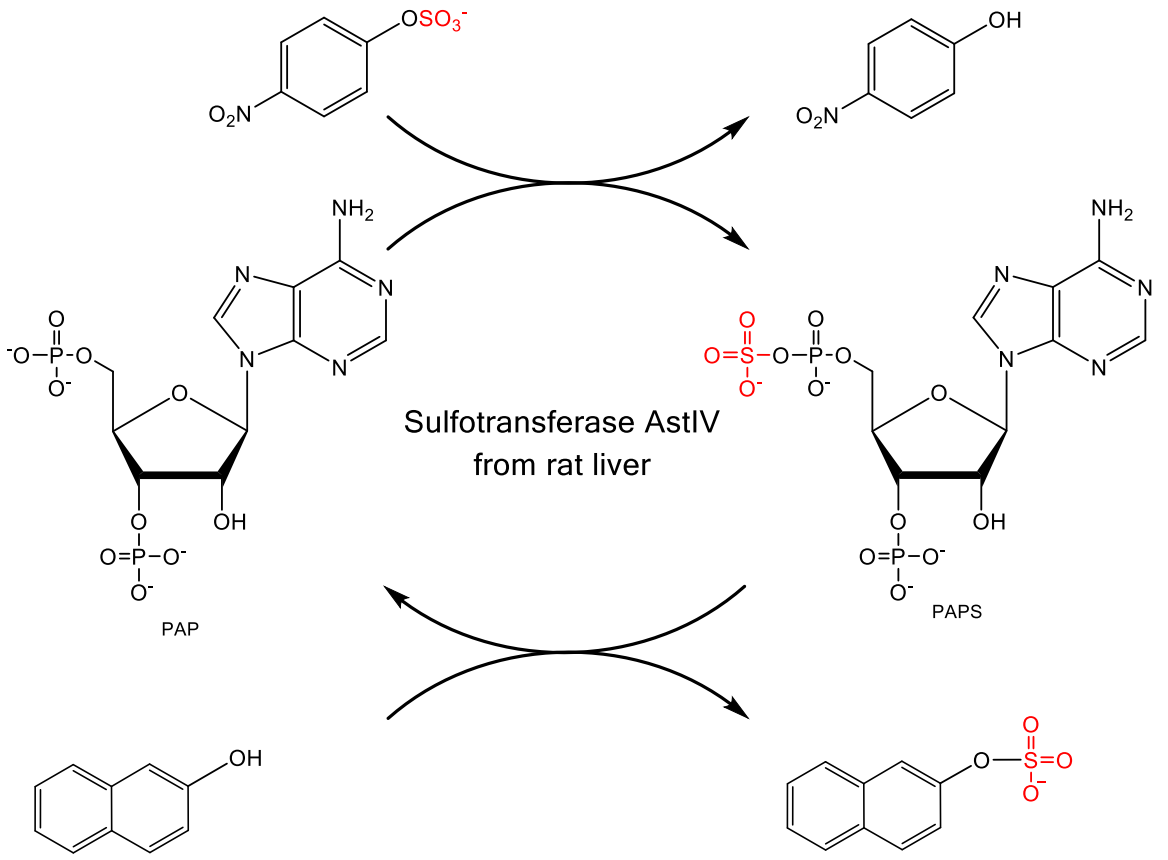

Figure 4.1: *Aryl sulfotransferases catalyzes the transfer of a sulfate group from phenolic sulfate esters (4-nitrophenyl sulfate) to a phenolic acceptor (2-naphthol) substrate* via *the PAPS cofactor system*[115,117].

4.2 Prokaryotic Sulfotransferases

Prokaryotic aryl sulfotransferases catalyze the transfer of a sulfuryl group from phenolic sulfate esters onto a phenol acceptor by a PAPS-independent mechanism[114]. Aryl sulfotransferases can be mostly found in the periplasm of strains of δ- and ε-proteobacteria[118]. There are a limited number of studies on this class of prokaryotic enzymes.

Depending on the origin of the bacteria, sulfotransferases are fundamental, e.g. in the biogenesis of secreted root-nodulation factors (signaling molecules between leguminous plants and bacteria)[119]; in the production of sulfated metabolites in mycobacteria, including the human pathogen *M. tuberculosis*[120]; in the sulfation of a variety of phenols (bacteria from human intestinal tract)[118]; in the sulfation of phenolic antibiotics (*Eubacterium* A-44)[121]; and in the detoxification pathway of phenolic compounds in rat liver (rat intestinal bacterium *Klebsiella* K-36)[122]. Aryl sulfotransferases exhibit a broad acceptor substrate specificity and thus the majority of the natural aryl sulfotransferases substrates and their physiologic roles remain unknown.

The first X-ray structure of an aryl sulfotransferase suggests that they differ substantially from all previously characterized sulfotransferases in their three-dimensional structure and active site architecture[123]. Two different classes of aryl sulfotransferases were described; it is likely that these classes have different reaction mechanism[124]. Class I contains proteins from protobacteria, showing high similarity with a conserved tyrosine residuum[125] in the active site and three conserved cysteine residues participating in catalytic activity[126,127]. In contrast, class II contains mainly proteins from firmicutes. Their sequences are less conserved, 30 residues longer in comparison to class I sequences, and they also contain eight cysteine residues[118]. The spatial arrangement of active site residues in bacterial aryl sulfotransferase is the same as that in PAPS-dependent aryl sulfotransferase[128].

Aryl Sulfotransferase from *Desulfitobacterium hafniense* (AST DH)

AST DH (EC 2.8.2.22) is a recombinant enzyme (ca 70 kDa) with a wide range of substrate specificity for phenolic and aliphatic substrates. According to sequence similarity AST DH belongs to class II of ASTs[127]. Various phenolic and aliphatic substrates were sulfated by this enzyme with coversion between 10 to 90 %[127,129]. In 2010 *astA* gene from *D. hafniense* was cloned in to pET26 vector, the plasmid containing AST DH was transformed to *E. coli* BL21(DE3) strain and expression of enzyme was observed after 16 hours at 25 °C. AST DH was shown to be the most active at pH 9.75 and 30 °C.

5 Aims of the Study

- to find enzyme(s) suitable for sulfation of selected flavonolignans and flavonols

- to express the aryl sulfotransferases from rat liver and *D. hafniense* in *E. coli*

- to use these aryl sulfotransferases in the preparation of sulfated metabolites of silybin (A&B; **1**&**2**), silybin A (**1**), silybin B (**2**), isosilybin A (**3**), isosilybin B (**4**), 2,3-dehydrosilybin (A&B; **5**&**6**), silychristin A (**7**), silydianin (**8**), quercetin (**9**), taxifolin (**10**), isoquercitrin (**11**) and rutin (**12**)

- to isolate, purify and fully characterize these metabolites

6 Results and Discussion

The major aim of this thesis was to prepare and fully characterize so far unknown sulfated metabolites of flavonoids, e.g. silybin (**1**,**2**), isosilybin (**3**,**4**), 2,3-dehydrosilybin (**5**&**6**), silychristin A (**7**, 95:5 silychristin B), silydianin (**8**), quercetin (**9**), taxifolin (**10**), isoquercitrin (**11**) and rutin (**12**). Recombinant aryl sulfotransferase IV from rat liver (AST IV) and from *D. hafniense* (AST DH) were chosen as representatives of mammalian and bacterial enzymes, respectively. The sulfated molecules prepared by a bacterial enzyme can be considered as the Phase II metabolites only when their structure is identical with products obtained by mammalian sulfotransferase. Alternatively the structure(s) can be confirmed by comparison with published *in vivo* studies. The exact identification of respective flavonoid conjugates from *in vivo* studies has been so far accomplished typically by HPLC-MS, MS or ^{1}H NMR analyses[14,16,40,78,130-132]. MS techniques, namely in polyphenols with multiple OH groups, allow to determine the number of conjugate groups but hardly their position(s). NMR techniques (besides X-ray spectroscopy) are the only methods able to describe a detailed structure. However, even this method is not trivial as the position of sulfate substitution can be determined only indirectly (no couplings available) from the shifts of the carbon atoms in the aromatic ring. Therefore, no reliable structures of the metabolites (conjugates) of taxifolin, rutin, isoquercitrin, silychristin, silydianin, silybin, isosilybin, dehydrosilybin were published so far.

6.1 Preparation of Enzymes Applied in this Study

6.1.1 Rat Liver Aryl Sulfotransferase IV (EC 2.8.2.1) (AST IV)

Cloning, Expression and Production of Recombinant AST IV

The gene of the *AstIV* from *Rattus norvegicus* (obtained from prof. L. Elling, RWTH Aachen, DE) was cloned into the pET16b expression vector *via* the 5′-*NdeI* and 3′-*BamHI* restriction sites[133] (Appendix 2). Transformation of obtained plasmid into following *E. coli* strains: BL21(DE3)Gold, BL21(DE3)Stars, BL21, BL21plyS, Origami(DE3) under various expression conditions was performed (*see tables* ***6.1*** *and* ***6.2***)[134] (Appendix 3). The expression of AST IV in *E. coli* BL21 clones was unsuccessful. Partial expression was observed (SDS-PAGE) in BL21(DE3)Stars clones, but the protein was entirely inactive.

Strong expression of AST IV was achieved in *E. coli* clones BL21(DE3)Gold (0.1 mM IPTG)[133] (Appendix 2).

Table 6.1: Conditions for AST IV expression: liquid medium and temperature.

***E. coli* strain**	**Medium**		**Temperature [°C]**		
	LB	TB	30	25	16
BL21	-	-	-	-	-
BL21(DE3)Gold	••	••	•	•	••
BL21(DE3)Star	•	•	•	•	•

(-) no expression and no activity of respective clone, (•) only expression, (••) expression and activity of respective clone were observed.

Clones BL21(DE3)Gold, BL21(DE3)Stars**,** BL21(DE3)plyS, Origami(DE3) were cultivated under the optimized conditions - TB medium, 16 °C and the enzyme was harvested 16 hours after induction (*table **6.2***). Weak activity of AST IV was detected in the clones BL21(DE3)plyS and Origami(DE3) (0.4 mM IPTG). The *E. coli* clones BL21(DE3)Gold were found to be the only suitable ones for the sulfation of silybin B (**2**), which was used as a representative of the studied compounds. *E. coli* BL21(DE3)Stars, BL21(DE3)plyS and Origami(DE3) clones were unable to sulfate silybin B (and compounds **1**, **1**&**2** as well)[134] (Appendix 3).

Co-transformation with chaperon G7 (GroEL/GroES) was tested for *E. coli* strains (BL21(DE3)Gold, BL21(DE3)plyS and Origami(DE3) to enable proper enzyme folding and to enhance AST IV activity. However, no significant improvement of the enzyme activity and stability was observed[134] (Appendix 3).

Table 6.2: Optimization of silybin B sulfation.

***E. coli* strain**	**Concentration of inductor (IPTG) [mM]**			**Sulfation of silybin B [conversion %]**
	0.1	0.4	0.8	
BL21(DE3)Gold	••	••	•	10
BL21(DE3)Star	•	•	•	N.D.
Origami(DE3)	••	••	•	0
BL21(DE3)plyS	•	••	•	0

(•) expression only, (••) expression and activity were observed. Red dots highlight the best cultivation conditions used in the sulfation of silybin B.

Purification of Recombinant AST IV

Purification *via* His-Tag employing Ni^{2+}NTA-agarose failed due to complete loss of activity (3 U/ml) plausibly due to high imidazole concentration (300 mM). Gel permeation chromatography (Superdex 200) proved to be suitable for a high-yielding AST IV purification[133] (Appendix 2).

Storage of Recombinant AST IV

Recombinant sulfotransferase IV suffers from low stability; during the 6-hour reaction at 37 °C the enzyme completely lost its activity; after one day at 4 °C or four days at -20 °C the enzyme lost half of its original activity. Numerous attempts to stabilize the enzyme and to enhance its activity were performed. The addition of 10 % glycerol (v/v) was found to be the only suitable stabilization method, which doubled the enzyme stability[133] (Appendix 2).

The best expression was achieved with *E. coli* BL21(DE3)Gold clones (without chaperones) cultivated under optimized conditions[134] (Appendix 3).

6.1.2 Aryl Sulfotransferase from *Desulfitobacterium hafniense* (AST DH)

Expression, Transformation, Cultivation and Purification of AST DH

Expression, purification and the (thermo)stability of aryl sulfotransferase from *D. hafninese* at different pH-values were described by van der Horst who found this enzyme to be quite stable; the addition of DMSO (10-25 %) or acetone (10 %) into the reaction mixture did not influence its activity[127]. However, the most challenging part of the present work was to reproduce van der Horst´s procedure[127]. Transformed cells (a kind gift of Prof. Hartog, University of Amsterdam, NL) were not as active as reported previously, and thus, a plasmid containing gene for AST DH was isolated and transformed again. A series of *E. coli* strains: BL21, BL21(DE3)Gold, BL21(DE3)plyS was transformed. Bacterial cultures were grown in LB medium with kanamycin and a second antibiotics (according to the respective *E. coli* strain) for improved selection and stabilization of the plasmid in the cells. The purity of the enzyme was confirmed by SDS–PAGE analysis, showing a single band of 70 kDa. The *E. coli* BL21(DE3)Gold clones were the most active. We found that the purification of this enzyme is not necessary as the respective vector strain is void of its own sulfatase activity. All these optimizations significantly improved the specific activity more than 250 fold (28 500 U/ mg)[134] (Appendix 3).

6.2 Sulfation of Silymarin Flavonolignans

6.2.1 Sulfation of Silymarin Flavonolignans Catalyzed by Recombinant AST IV

Pure silybin A (**1**), silybin B (**2**) and natural silybin (**1**&**2**) were the first flavonolignan substrates, which were sulfated by AST IV. Since silybin is poorly soluble in water, co-solvents were required for the enzymatic reactions. Significant loss of the activity and stability of AST IV after adding 20 % of organic co-solvent (ethanol or acetone) was observed. 10 % DMSO notably increased the solubility of silybin (1 mg/ml) without

affecting the enzymatic reaction. Fresh enzyme (0.4 ml) had to be supplemented twice a day to keep the reaction running. Hence, we overcame poor stability of AST IV and the conversion of 92 % was reached in 5 days *(Fig. **6.1**).* Reaction required an addition of PAP as a cofactor[133] (Appendix 2).

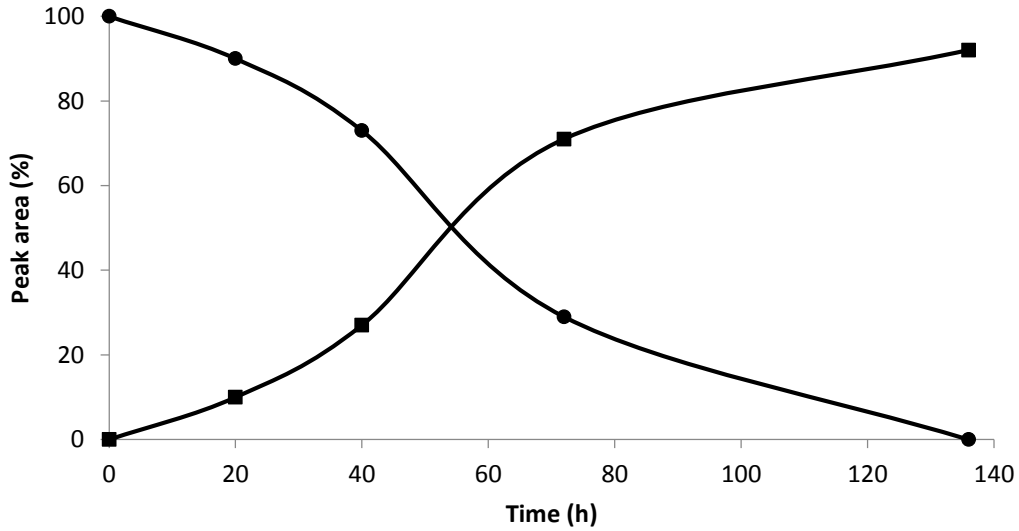

Fig. 6.1: *Sulfation of silybin B by p-nitrophenyl sulfate catalyzed by purified AST IV (total volume 1.5 ml). Silybin B (●) and silybin B 20-*O*-sulfate (■).*

AST IV was found to be strictly stereoselective as it sulfated exclusively silybin B (**2**); silybin A (**1**) was not the substrate for this enzyme.

Problems with purification and stability of AST IV, regeneration of the cofactor (PAP) and scale-up prompted us to develop an entirely new concept employing whole *E. coli* cells transformed with the respective gene. The reaction mixture (preparatory scale) typically contained the substrate (**1**, **2**, **7**, **8** respectively, 40 mg), 4 g (wet based) of freshly prepared and buffer-washed *E. coli* cells (BL21(DE3)Gold strain), *p*-NPS and 4 ml of DMSO. The reaction mixture was incubated at 37 °C for 24 hours; the fresh cells were added three times during the reaction course to keep the reaction progress[133] (Appendix 2, Appendix 5).

The only sufficient conversion of all tested substrates (**1-2, 7, 8**) was observed for silybin B (up to 48 %) and silybin B 20-*O*-sulfate was isolated (**2a**, 10 mg, 25 %, Appendix 2)[133]. *Table **6.3*** sums up the formation of sulfated products of respective substrates (**1**, **2**, **7**, **8**, Appendix 5).

Table 6.3: *Overview of AST IV substrate specificity and isolated sulfated products.*

Substance	Formation of *p*-NP	Isolated product(s)	Product structure
Silybin (**1**&**2**)	●	○	silybin B 20-*O*-sulfate (**2a**)
Silybin A (**1**)	-	-	-
Silybin B (**2**)	●	●	silybin B 20-*O*-sulfate (**2a**)
Silychristin A (**7**)	●	N.D.	-
Silydianin (**8**)	●	N.D.	-

(-) compound is not a substrate for AST IV; (●) isolated sulfated product(s) and/or release of p-NP; (○) the case of natural silybin, only silybin B sulfate was isolated. Isosilybin and 2,3-dehydrosilybin were not tested due to their paucity.

At this point we could confirm our previous observations that silybin A is completely inert to sulfation with mammalian sulfotransferase. Moreover, the experiment performed on the natural silybin (mixture of A&B, *Fig.* ***6.2***) might be considered as an indirect proof that silybin A is not an inhibitor of AST IV, because we observed same conjugation rate of silybin B in this case as with the pure silybin B. Therefore, it is obvious that each silybin diastereoisomer has its own specific route of Phase II metabolism[37,40]. We can speculate that it is probably due to the differences in their structures. Silybin B is planar *(Fig.**6.3***) while the molecule of silybin A is curved *(Fig.* ***6.3****)*, and probably does not fit well into the active site of AST IV[133] (Appendix 2). When silychristin A (**7**) or silydianin (**8**) were tested as substrate in this AST IV catalyzed reaction both consumption of *p*-NPS and formation of *p*-nitrophenol (*p*-NP) were observed. This finding clearly proved the sulfation reactions. Despite our attempts no sulfated products were isolated and recovery of the substrate was impossible. We assume that these substrates were probably sulfated, but immediately metabolized by the cells, hence the sulfation products could not be isolated. Isosilybin diastereomers (**3**, **4**) and 2,3-dehydrosilybin (mixture **5**&**6**) were not tested due to their paucity (Appendix 5).

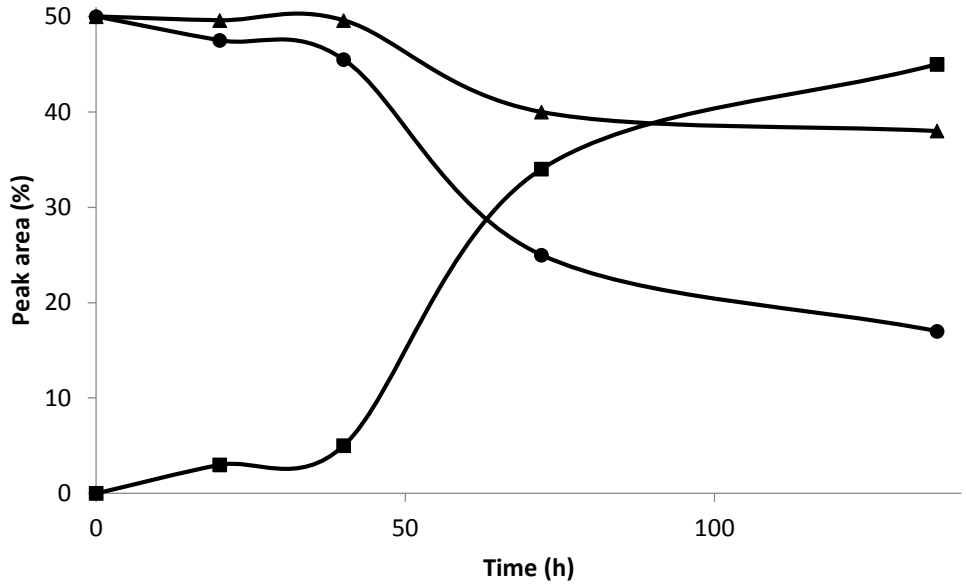

Fig. 6.2: *Sulfation of natural silybin catalyzed by whole cells transformed with AST IV. Silybin B* (●)*, silybin A* (▲) *and silybin B 20-O-sulfate* (■).

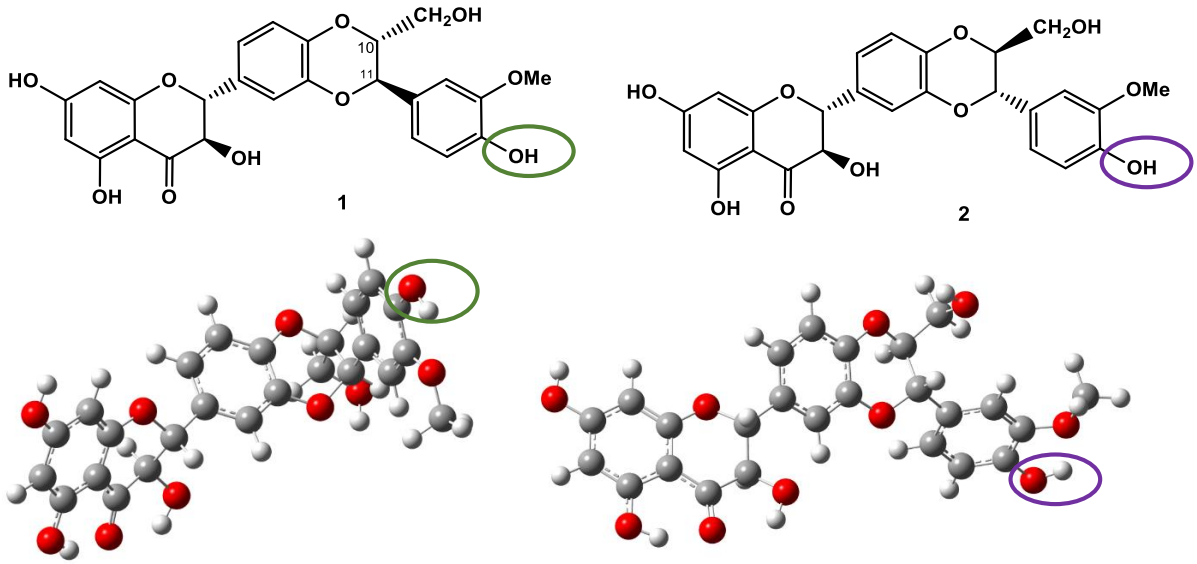

Fig. 6.3: *Position of sulfation catalyzed by AST IV displayed on the structure of silybin A (**1**) and silybin B (**2**) and their 3D models. Circles show the position of sulfation.*

6.2.2 Silybin and Isosilybin Sulfation with Purified AST from *D. hafniense*

The optically pure silybin A (**1**) and B (**2**), isosilybin A (**3**) and B (**4**) were in the first instance successfully sulfated by purified AST DH. However, this enzyme preparation had poor specific activity (up to 10 U/ mg) and sulfation of flavonolignans required long time (one week for 100 % conversion). The stereochemistry of respective substrates strongly influenced the reaction kinetics analogously to the rat enzyme AST IV. Isosilybin A was

conjugated faster and more efficiently than isosilybin B. Similarly silybin B was conjugated to a larger extent than silybin A. After isolation and purification (*see chapter 6.3*), silybin A 20-*O*-sulfate (**1a**, 20 mg, 58 %), silybin B 20-*O*-sulfate (**2a**, 22 mg, 62 %), isosilybin A 20-*O*-sulfate (**3a**, 14.5 mg, 57 %), isosilybin B 20-*O*-sulfate (**4a**, 11.3 mg, 50 %) were obtained and fully characterized[135] (Appendix 1).

6.2.3 Sulfation of Silymarin Flavonolignans Catalyzed by Crude Lysate of AST DH

Due to the enhanced sulfation activity of AST DH crude extract, the reaction time was reduced from one week to ca 4 hours reaching nearly quantitative conversion. Acetone was found to be most promising co-solvent in this case, as it did not significantly reduce the activity of the lysate[127], it dissolves all silymarin flavonolignans and replacing DMSO by acetone also enabled easier TLC monitoring. At pH 8.9 silymarin substrates (but also taxifolin) were apt to air oxidation forming thus respective dehydro-derivatives and their sulfates. Oxidation can be limited by using inert atmosphere (Ar) and shorter reaction time. *Table **6.4*** sums up the optimized reaction conditions, which were applied in all sulfation reactions forming the sulfated products: silybin A&B 20-*O*-sulfate (**1a**&**2a**, 125 mg, 60 %, starting from mixture **1**&**2**, 1:1), silybin A 20-*O*-sulfate (**1a**, 120 mg, 62.5 %), silybin B 20-*O*-sulfate (**2a**, 135 mg, 67.5 %), 2,3-dehydrosilybin A&B 20-*O*-sulfate (**5a**&**6a**, 10 mg, 25.6 %, starting from mixture **5**&**6**), 2,3-dehydrosilybin A&B 7,20-*O*-disulfate (**5b**&**6b**, 9 mg, 28.6 %, starting from mixture **5**&**6**); silychristin A 19-*O*-sulfate (**7a**, 100 mg, 57.1 %); silydianin 20-*O*-sulfate (**8a**, 90 mg, 75.4 %) (Appendix 5).

Table 6.4: Optimized reaction conditions for sulfation of substrates (**1-8**) by AST from *D. hafniense* (AST DH).

Reaction condition	**Original procedure (purified enzyme)**	**Optimized procedure (crude cell lysate)**
Amount of substrate [mg]	30	≥150
Co-solvent	DMSO	Acetone
Amount of catalyst [ml]	0.250	2
Atmosphere	Air	Argon
Reaction time [h]	160	4
Reaction monitoring	HPLC	TLC, HPLC

6.3 Purification of Sulfated Flavonolignans

The sulfated products **1a** – **8a** contain highly polar sulfate group(s) and thus the whole molecule becomes very polar. This feature brings a number of beneficial properties, such as good water-solubility or possibly better bioavailability. On the other hand, polar or amphiphilic nature of these compounds can cause serious purification problems. A fundamental problem was a large amount of *p*-NP in the reaction mixture, which needed to be removed. A series of classical laboratory purification processes such as column chromatography, preparative TLC, precipitation, extraction, solid phase extraction (SPE), gel chromatography, preparative HPLC or crystallization techniques were tested. Solid phase extraction (Chromabond C-18; SPE) was chosen as the purification method of choice for sulfated products **1a**, **2a**, **3a** and **4a**. This method has several advantages. It is fast, efficient, and product and substrate were eluted separately by a gradient of methanol[135, 133] (30 % sulfated product, 100 % substrate, Appendix 1 and 2). Alternatively, **1a**, **2a**, **3a** and **4a** could be repetitively extracted with *n*-butanol and purified by column chromatography[135] (Appendix 1). However, both these procedures have some drawbacks, e.g. poorer performance in larger volumes and quantities (up to 5 ml) and inefficiency in purification of some sulfated products such as silydianin sulfate, silychristin sulfate or flavonol sulfates.

When silychristin A and silydianin sulfates were prepared by whole cell biotransformation the preparative HPLC using Asahipak column was used as a pre-purification step. Then fractions containing putative sulfated product (according to HPLC) were re-purified by gel chromatography (Sephadex LH-20; 80 % methanol).

In a narrow pH range 7.5 – 7.7 the *p*-NP could be easily removed by extraction with ethyl acetate, while the sulfated product remained in the aqueous phase. Gel chromatography employing Sephadex LH-20 (80 % methanol) followed the ethyl acetate extraction led to high purity (>95 %) and high yields (60 %) of all sulfated products (**1a-8a**) in the preparatory scale. *Table **6.5*** sums up the purity and yields of isolated and purified sulfated products of respective silymarin flavonolignans[133, 135] (Appendix 1, 2 and 5).

Table 6.5: Sulfated products **1a-8a.**

Products	**Method**	**Yield [%]**
Silybin B 20-*O*-sulfate (**2a**)	AST IV	25
Silybin (A&B) 20-*O*-sulfate (**1a**&**2a;** 1:1)	AST DH	60
Silybin A 20-*O*-sulfate (**1a**)	AST DH	63
Silybin B 20-*O*-sulfate (**2a**)	AST DH	68
Silybin A 20-*O*-sulfate (**1a**)	AST DH*	58
Silybin B 20-*O*-sulfate (**2a**)	AST DH*	60
Isosilybin A 20-*O*-sulfate (**3a**)	AST DH*	57
Isosilybin B 20-*O*-sulfate (**4a**)	AST DH*	50
2,3-Dehydrosilybin (A&B) 20-*O*-sulfate (**5a**&**6a**)	AST DH	26
2,3-Dehydrosilybin (A&B) 7,20-*O*-disulfate (**5b**&**6b**)	AST DH	29
Silychristin A19-*O*-sulfate (**7a**)	AST DH	57
Silydianin 20-*O*-sulfate (**8a**)	AST DH	75

(*) purified AST DH

6.4 Characterization of Flavonolignan Sulfates

The major problem in sulfate characterization is the identification of sulfate group position. The presence of these groups in flavonoids (and flavonolignans as well) can be either confirmed by infrared spectroscopy - strong bands at 1200 (S=O) and 1040 (C-O-S) cm^{-1} or by enzymatic hydrolysis employing aryl sulfatases[136]. MS spectroscopy typically gives information on the number of substitutions, but little information about their position(s) (with few exceptions). Hence, the structure determination of all sulfated products (**1a-8a**) was accomplished by a combination of MS, ^{1}H-, ^{13}C-NMR experiments (concerning MS/MS fragmentation[135] (Appendix 2), COSY, HSQC[133, 135] (Appendix 1, 2 and 5). The position of the sulfated group was identified to be at the catechol moiety for each flavonolignan derivative (**1a-8a**, *Fig.* ***6.4***). The subtraction of the spectra of flavonolignans from the ones of their sulfated metabolite revealed typical patterns of carbon chemical shifts on the catechol moiety (Appendix 5).

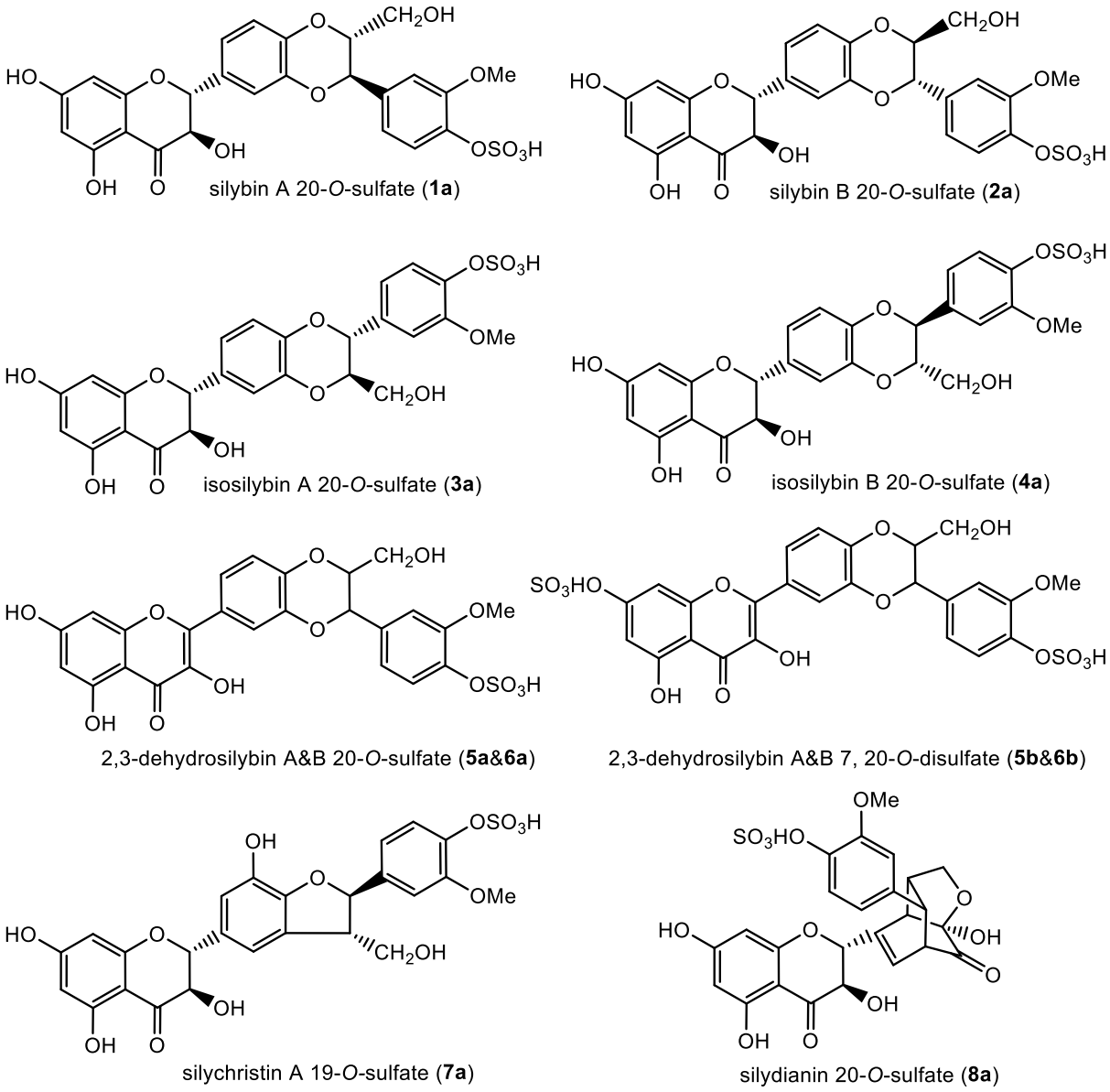

Fig. 6.4: *Structures of all fully characterized sulfated flavonolignans*

6.5 Sulfation of Silybin Synthetic Derivatives Catalyzed by AST DH

Not only natural substrates **1**-**8** can be utilized by this enzyme forming respective sulfates (**1a-8a**), but also some selectively substituted silybin derivatives can be converted to their sulfated forms. Silybin 20-*O*-sulfate, silybin 7-*O*-butyrate, silybin 7-*O*-glucuronide, silybin 7,23-*O*-disulfate, 20-*O*-methyl silybin, silybin 23-*O*-monovinyldodecanoate, silybin 23-*O*-acetate and silybin 23-*O*-butyrate were sulfated according to the described sulfation procedure. Reactions were monitored by HPLC for 24 hours. Preliminary results suggest that substituents at C-7-OH of silybin do not influence the reaction at all: silybin 7-*O*-butyrate or silybin 7-*O*-glucuronide were fully converted to their sulfated form. Free

C-20 OH of silybin was found to be crucial for the sulfation reaction. Its substitution blocked sulfation of the molecule (substrates silybin 20-*O*-sulfate and 20-*O*-methyl silybin). Not entirely uniform results were achieved with silybin substituted at 23-OH. Silybin 23-*O*-acetate and silybin 23-*O*-butyrate were sulfated, but no sulfation of silybin 7,23-*O*-disulfate or silybin 23-*O*-monovinyldodecanoate was observed (Appendix 5).

6.6 Sulfation of Flavonols

6.6.1 Sulfation of Flavonols Catalyzed by Resting Cells Transformed by AST IV

A further goal of this study was to prepare and fully characterize sulfates of the flavonoid substrates: quercetin (**9**), taxifolin (**10**), isoquercitrin (**11**) and rutin (**12**). We employed our previously described procedures using resting microbial cells harboring AST IV[135] (Appendix 2). Since our original procedure was not feasible and solubility of these substrates is better, we slightly optimized the reaction conditions[134] (Appendix 3). The cells were removed once a day and newly prepared cells (2 grams, wet weight) were supplemented each day within 3 days. The reaction time was prolonged but it significantly improved the work with the reaction mixture. The formation of sulfated products was monitored by HPLC and after 72 hours the reaction was stopped. The sulfated products were then purified.

The release of *p*-NP indicated the reaction progress, followed by respective sulfated product formation (*table **6.6***). In the case of quercetin, formation of *p*-NP clearly showed that sulfation reaction occurred, however, despite numerous attempts no sulfated product could be isolated. We assume that quercetin, similarly as silydianin and silychristin A, was probably sulfated but subsequently metabolized by the cells, thus the sulfation reaction could be proved only indirectly. Glycosylated derivatives of quercetin, e.g. isoquercitrin (**11**) and rutin (**12**) were not substrates of this enzyme. This finding clearly corresponds with literature data[78], e.g. isoquercitrin is at first deglycosylated to quercetin and this is then further conjugated. Taxifolin (**10**) was the only substrate accepted by AST IV yielding taxifolin monosulfate **10a** (1 mg, 1.5 %), which was characterized only by HRMS due to its paucity. We assume that it is 3′-*O*-taxifolin sulfate (**10a**), since another isolated product from this reaction was identified to be 3′-*O*-quercetin sulfate **9a** (8 mg, 12 %), which is plausibly an oxidation product of **10a**. Proposed oxidation with cytochromes was ruled out using a respiration inhibitor NaN_3 – and the same products were obtained. We could not distinguish whether the oxidation of taxifolin skeleton occurred in form of intact taxifolin or in the form of its sulfate. Nevertheless, results obtained with quercetin suggest that taxifolin was sulfated

first and then the sulfated product was oxidized to the respective quercetin sulfate[134] (Appendix 3).

Table 6.6: Overview of AST IV substrate specificity towards flavonoids (quercetin and its derivatives).

Substance	Formation of *p*-NP	Isolated product(s)	Characterized product
Taxifolin	●	●	taxifolin 3′-*O*-sulfate (**10a**) quercetin 3′-*O*-sulfate (**9a**)
Quercetin	●	N.D.	-
Isoquercitrin	- (*)	-	-
Rutin	- (*)	-	-

*(-) no product was isolated, * this compound is not a substrate for AST IV; (●) isolated sulfated product(s) and/or release of p-NP.*

6.6.2 Sulfation of Flavonols Catalyzed by AST DH

Due to the failure of our previous attempts to isolate and characterize sulfated quercetin, isoquercetin and rutin by AST IV from rat liver, the sulfation reaction had to be repeated with AST from *D. hafninese* (AST DH). We followed our previously optimized procedure (*see chapter 6.2.3*). Starting from the substrates **9**-**12**, following products were obtained: isoquercitrin 4′-*O*-sulfate (**11a**, 120 mg, yield 69 %), rutin 4′-*O*-sulfate (**12a**, 90 mg, yield 53 %), unseparable mixture of taxifolin 4′-*O*-sulfate (**10b**) and taxifolin 3′-*O*-sulfate (**10c**, side product, ca 16 %,) (190 mg, yield 75 %), mixture of quercetin 3′-*O*-sulfate (**9a**) and quercetin 4′-*O*-sulfate (**9b**, side product, ca 25 % separable only by HPLC, 90 mg, yield 47 %)[134,137] (Appendix 3 and 4).

6.6.3 Kinetics of Regioisomer Sulfate Formation of Taxifolin and Quercetin

Formation of hardly separable mixture of taxifolin sulfates (**10b** and **10c**) and quercetin sulfates (**9a** and **9b**) provoked further investigation in this field, and therefore, kinetics of taxifolin and quercetin regioisomer sulfate formation was studied. We have found that quercetin 3′-*O*-sulfate (**9a**) is the major product only when the reaction time is longer than 4 hours with high dose of AST DH (360 U/ ml). Lower enzymatic activity led to the preferential production of quercetin 4′-*O*-sulfate (**9b**), but both regiosomers were always present in the reaction mixture. On the other hand, the same reactions performed with taxifolin revealed that reaction time and enzymatic activity do not influence the ratio

between taxifolin sulfate regioisomers. Taxifolin 4′-*O*-sulfate (**10b**) was always the major product (80 %)[134] (Appendix 3).

6.7 Purification and Characterization of Flavonoid Sulfates

All listed compounds were purified by ethyl acetate extraction at pH 7.5 – 7.7 followed by chromatography on Sephadex LH-20[134] (Appendix 3). *Table **6.7*** sums up the purity and yields of isolated and purified sulfated products that were characterized by MS and NMR (except **10a** - only HRMS was measured due to its paucity).

Table 6.7: Sulfated flavonoids **9a-12a**.

Products	**Yield [%]**	**Yield [mg]**
Taxifolin sulfate (mixture of **10b** and **10c**, 80:20)	75	190
Taxifolin sulfate (**10a**)*	1	1.5
Quercetin sulfate (**9a**)*	8	12
Quercetin sulfate (mixture of **9a** and **9b**, 75:25)	47	90
Isoquercitrin sulfate (**11a**)	69	120
Rutin sulfate (**12a**)	53	90

(*) obtained by whole cell biotransformation (AST IV).

There is a lack of structural information about flavonoid metabolites in general. Particularly the sulfates have not been researched thoroughly[138-141] or there are numerous controversies in the literature. Quercetin metabolites were previously characterized only to some extent, a proper identification and structure elucidation of the flavonoid sulfates produced in the present work involved a complex study. Sulfate was present at the catechol moiety of each flavonol derivative (*Fig. **6.5***). However, the exact determination of its position was particularly complicated. The identification of the sulfation position required methylation of the sulfated product as accomplished at the molecule of isoquercitrin sulfate (**11a**). Methylation caused narrowing and focusing of respective NMR signals and this enabled exact structure determination and all sulfated products **9a, 9b, 10a, 10b, 11a, 12a**. The structure determination was achieved by the combination of HRMS, ^{1}H, ^{13}C NMR experiments including COSY, HSQC[134] (Appendix 3). As in the case of other sulfated metabolites the shifts in carbon spectra revealed typical patterns after subtraction of the spectra of respective parent compound from that of the respective sulfate. The results

obtained in this study are in contrast with previous findings[129,142], which were found to be mistaken[134] (Appendix 3). These methods provide generally applicable methodology of determination of sulfate (or another group without direct NMR interactions) at the compounds bearing catechol moiety.

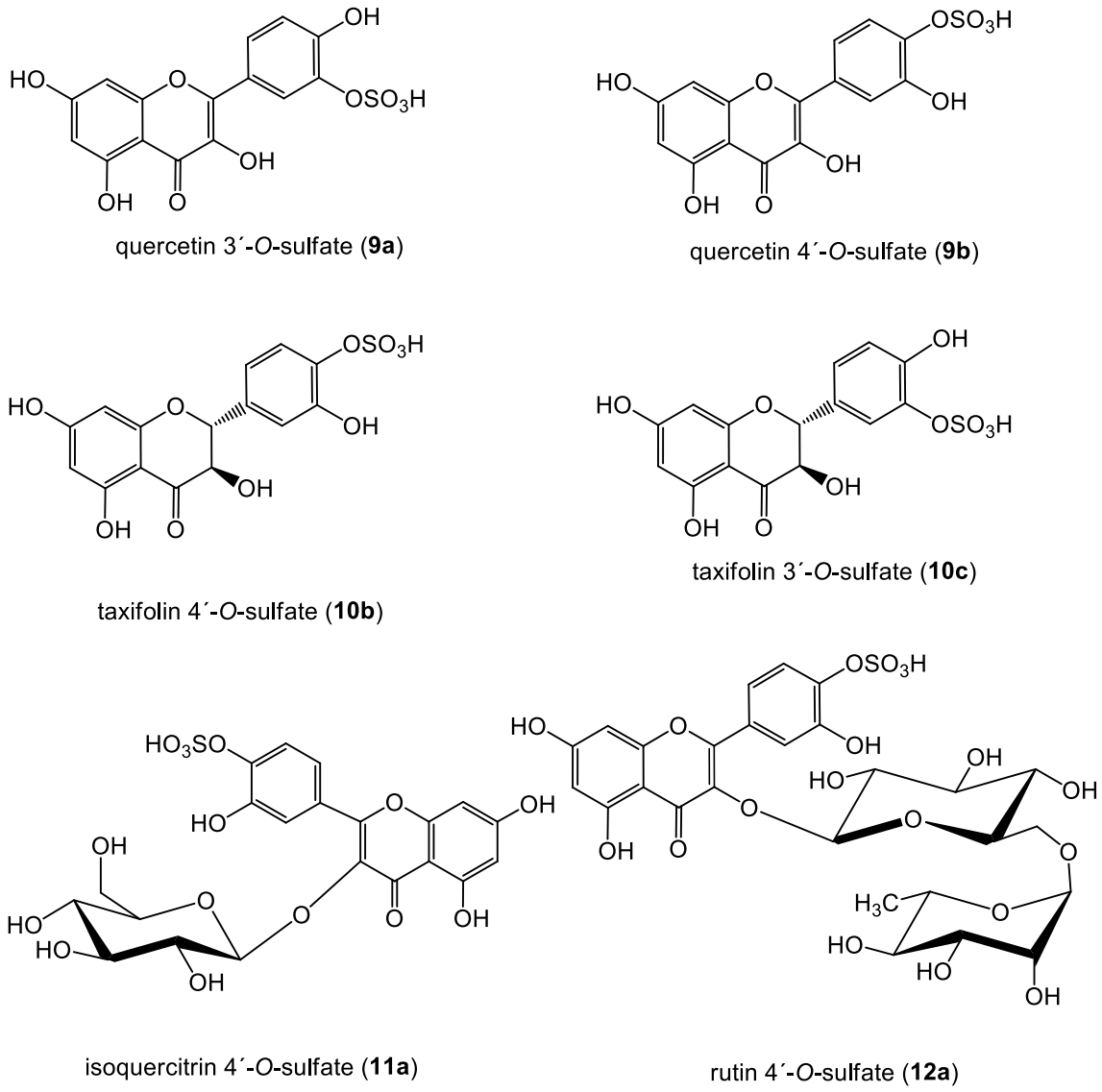

Fig. 6.5: *An overview of all isolated and fully characterized flavonoid sulfates.*

6.8 Comparison of Studied Enzymes

AST IV from rat liver is not a suitable catalyst for sulfate preparation as it is a time demanding and low yielding enzyme. On the other side, the use of this enzyme provides some information on the structure of the authentic sulfated metabolites in mammals.

In contrast, AST from *D. hafniense* (AST DH) was identified as a perfect tool for the sulfation of numerous substrates – it is an efficient and high-yielding enzyme. Moreover, we found that this enzyme produces identical regioisomeric sulfated derivatives as the mammalian enzyme. *Table **6.8*** sums up the properties of both catalysts[134] (Appendix 3).

Table 6.8: Comparison of two studied AST reaction and the catalyst properties.

Properties	**AST IV from rat liver**	**AST DH from *D. hafniense***
PAP needed	yes	no
Catalyst	whole cells	lysate
Optimum pH	7	9
Optimum temperature (°C)	37	30
Reaction time	3 days	4 hours
Co-solvent	DMSO	acetone
Yield	0-25 %, (10 mg)	50-70 %, (100 mg)
Catalyst efficiency (ml of LB media needed for 1 mg of product)	450	0.1

7 Conclusions

The recombinant aryl sulfotransferase IV from rat liver (AST IV) and recombinant aryl sulfotransferase from *D. hafniense* (AST DH) were employed in sulfation reaction of flavonolignans and flavonoids; namely silybin A and silybin B, isosilybin A and isosilybin B, 2,3-dehydrosilybin, silychristin A, silydianin, quercetin, taxifolin, isoquercitrin and rutin.

Recombinant AST IV from rat liver was expressed in *E. coli* BL21(DE3)Gold cells and purified. This enzyme was very unstable. The original activity was low (3 U/ml), it was decreased rapidly and it performed poorly in larger volumes. Thus *E. coli* cells transformed with AST IV were employed in sulfation reactions.

The *E. coli* BL21(DE3)Gold clones harboring AST DH were found to be the most active. Purification of this enzyme was not necessary due to non-detectable sulfatase activity. Such optimization of production method significantly improved the specific activity more than 250 fold (28 500 U/mg). Crude cell lysate containing AST DH could be used for sulfation reaction.

The reaction conditions for each enzyme were optimized: for purified AST IV, purified AST DH, the whole cell biotransformation (AST IV) and crude cell lysate containing AST DH. When the resting *E. coli* cells expressing AST IV catalyzed the sulfation reaction, the only sufficient conversion of all tested substrates was observed for silybin B. Silybin B 20-*O*-sulfate was isolated, purified with satisfactory yield and characterized. Taxifolin yielded small amount of taxifolin 3′-*O*-sulfate and quercetin 3′-*O*-sulfate. In the case of silychristin A, silydianin and quercetin formation of *p*-NP was detected, but no sulfated products were isolated. Silybin A, isoquercitrin and rutin were not transformed by this enzyme.

Silybin A, silybin B, isosilybin A and isosilybin B were transformed by purified AST DH to silybin A 20-*O*-sulfate, silybin B 20-*O*-sulfate, isosilybin A 20-*O*-sulfate, isosilybin B 20-*O*-sulfate, which were fully characterized by MS and NMR.

Sulfation of silybin, silychristin A, silydianin, 2,3-dehydrosilybin, quercetin, isoquercetin and rutin by AST DH (crude lyzate) yielded silybin A&B 20-*O*-sulfate, silybin A 20-*O*-sulfate, silybin B 20-*O*-sulfate, 2,3-dehydrosilybin 20-*O*-sulfate, 2,3-dehydrosilybin 7,20-*O*-disulfate, silychristin A 19-*O*-sulfate, silydianin 20-*O*-sulfate, isoquercitrin 4′-*O*- sulfate, rutin 4′-*O*-sulfate, unseparable mixture of taxifolin 4′-*O*-sulfate and taxifolin 3′-*O*- sulfate, mixture of quercetin 3′-*O*-sulfate and quercetin 4′-*O*-sulfate.

The purification procedures for each method of preparation and HPLC characterization for each sulfated product were developed. All isolated compounds were characterized by

MS and NMR. Characteristic patterns in NMR spectra were identified for catechol 3′- and 4′-sulfates. These data will enable the identification of analogous flavonoid metabolites. The sulfated metabolites can be used for *in vitro* evaluation of biological activities (Appendix 4) and as authentic standards for metabolic studies *in vivo*.

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sulfates by sulfotransferase from *Desulfitobacterium hafniense*, *J Mol Catal B: Enzym 89*, 24-27.
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9 Supplementary data

List of publications

Articles related to the presented thesis

Appendix 1:

Marhol, P., Hartog, A.F., van der Horst, M.A., Wever R., **Purchartová, K.**, Fuksová, K., Kuzma, M., Cvacka, J., Křen,V.: Preparation of silybin and isosilybin sulfates by sulfotransferase from *Desulfitobacterium hafniense, J. Mol. Catal. B: Enzym*. 89, 24-7 (2013)

Appendix 2:

Purchartová, K., Engels, L., Marhol, P., Šulc, M., Kuzma, M., Slámová, K., Elling, L., Křen, V.: Enzymatic preparation of silybin phase II metabolites: sulfation using aryl sulfotransferase from rat liver. *Appl. Microbiol. Biotechnol.* 97, 10391-8 (2013)

Appendix 3:

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Appendix 4:

Roubalová, L., **Purchartová, K.**, Papoušková, B., Vacek, J., Křen, V., Ulrichová, J., Vrba, J.: Sulfation modulates the cell uptake, antiradical activity and biological effects of flavonoids *in vitro*: An examination of quercetin, isoquercitrin and taxifolin. *Bioorg. Med. Chem. 23*, 5402-09 (2015)

Appendix 5: (manuscript in preparation)

Valentová, K., **Purchartová, K**., Marhol, P., Pelantová, H., Cvačka, J., Biedermann, D., Křen, V. Prokaryotic and eukaryotic aryl sulfotransferases: Sulfation of unexplored flavonolignans from silymarin. *Int. J. Mol. Sci.*

All supplementary data to the articles1-4 are available online.

Reviews Related to the Presented Thesis

Appendix 6:

Theodosiou, E., **Purchartová, K.**, Stamatis, H., Kolisis, F., Křen V.: Bioavailability of silymarin flavonolignans: Drug formulations and biotransformation. *Phytochem. Rev.* 13, 1-18 (2013)

Appendix 7:

Křen, V., Marhol, P., **Purchartová, K.**, Gabrielová, E., Modrianský M.: Biotransformation of silybin and its congeners. *Curr. Drug Metab.* 14, 1009-21 (2013)

Other Research Articles

Purchartová, K., Marhol, P., Gažák, R., Monti, D., Riva, S., Kuzma, M., Křen, V.: Regioselective alcoholysis of silybin A and B acetates with lipases. *J. Mol. Catal. B: Enzym., 71*, 119-123 (2011)

Charrier, C., Azerad, R., Marhol, P., **Purchartová, K.**, Kuzma, M., Křen, V. Preparation of silybin phase II metabolites: *Streptomyces* catalyzed glucuronidation, *J. Mol. Catal. B: Enzym*. *102*, 167-173 (2014)

Vavříková, E., Gavezzotti, P., **Purchartová, K.**, Fuksová, K., Biedermann, D., Kuzma, M., Riva, S., Křen, V.: Regioselective alcoholysis of silychristin acetates catalyzed by lipases. *Int. J. Mol. Sci. 16*, 11983-11995 (2015)

Oral presentations:

Purchartová K.: Comparison of aryl sulfotransferases - metabolic studies of flavonoids and flavonolignans. 4th International Conference on Novel Enzymes, Gent, Belgium, (14.-17.10. 2014)

Purchartová K: Enzymová příprava, charakterizace a vlastnosti sulfatovaných derivátů flavonoidů a flavonolignanů. Doktorandský seminář, Charles University in Prague, Faculty of Science, Department of Biochemistry, Prague (23.11.2015)

Purchartová K.: Regioselective alcoholysis of silybin acetates with lipases. Annual seminar of project GACR 305/09/H008 „Preparation, biotransformation and optimization of compounds with antitumor and antimicrobial effects“, Nové Hrady, (9. – 11.6.2011)

Poster presentations

Purchartová K., Monti D., Gažák R., Marhol P.,Biedermann D., Riva S., Křen V.: Enzymatic kinetic resolution of silybin diastereoisomers. ESF-COST High-level research conference, Natural products chemistry, Biology and Medicine III, Aquafredda di Maratea, Italy (5.9. – 10.9.2010)

Purchartová K., Marhol P., Gažák R., Monti D., Riva S., Kuzma M., Křen V.: Regioselective alcoholysis of silybin A and B acetates with lipases, Biotrans 2011, Sicily, Italy (2. - 6.10. 2011)

Purchartová K., Marhol P., Biedermann D., Elling L., Křen V.: Silybin metabolites: Chemical and enzymatic preparation, 5th International Conference on Polyphenols and Health (ICPH), Sitges (Barcelona) Spain (17. - 20.10. 2011)

Purchartová K., Marhol P., Biedermann D., Engels L., Elling L., Křen V.: Enzymatic preparation of silybin metabolites, Biocat 2012, Hamburg, Germany (2. - 5.9. 2012)

Purchartová K., Marhol P., Hartog, A.F., van der Horst, M.A., Wever R., Křen V.: Preparation of silybin and isosilybin sulfates by sulfotransferase from *Desulfitobacterium hafniense,* 7th ISANH Congress on Polyphenols Applications, Bonn, Germany (6. - 7.6. 2013)

Purchartová, K., Engels, L., Marhol, P., Slámová, K.,Elling, L., Křen, V.: Enzymatic preparation of silybin metabolites: sulfation using aryl sulfotransferase from rat liver. Biotrans 2013, Manchester, United Kingdom, (21. - 25.7. 2013)

Purchartová K., Marhol P.,Wever R., Křen V.: Preparation of sulfated flavonoid metabolites by sulfotransferase from *Desulfitobacterium hafniense*, Biocat 2014, Hamburg, Germany (31.8. - 4.9. 2014)

Purchartová K., Valentová K., Křenková A., Marhol P., Pelantová H., Kuzma M., Cvaka J., Biedermann D., Křen V.: Preparation of sulfated metabolites of flavonoids and flavonolignans by arylsulfotransferase from *Desulfitobacterium hafniense*, 7th International Conference on Polyphenols and Health, ICPH 2015, Tours, France (27. - 30.10.2015)

Purchartová K., Valentová K., Křenková A., Marhol P., Pelantová H., Křen V.: Sulfation of quercetin and its derivatives, Biotrans 2015, Vienna Austria (26. - 30.7. 2015)

Purchartová K., Valentová K., Křenková A., Marhol P., Pelantová H., Kuzma M., Cvaka J., Biedermann D., Křen V.: Arylsulfotransferase from *Desulfitobacterium hafniense* for the preparation of sulfated metabolites of flavonoids and flavonolignans. 3rd WG Meeting of the COST Action FA1403 POSITIVe, Bucharest, Romania (15. - 17. 3. 2016)

External Fellowship

2012 COST STSM at National Food Institute, Technical University of Denmark Soltofts Plads Building 221, Kg. Lyngby 2800, Denmark, supervisor Dr. Caroline P. Baron

2014 Academic exchange with Istituto di Chimica del Riconoscimento Molecolare, via Mario Bianco 9, I 20131 Milano, supervisor Dr. Sergio Riva

Appendix 1:

Marhol, P., Hartog, A.F., van der Horst, M.A., Wever R., **Purchartová, K.**, Fuksová, K., Kuzma, M., Cvacka, J., Křen,V.: Preparation of silybin and isosilybin sulfates by sulfotransferase from *Desulfitobacterium hafniense, J. Mol. Catal. B: Enzym*. 89, 24-7 (2013)

Appendix 2:

Purchartová, K., Engels, L., Marhol, P., Šulc, M., Kuzma, M., Slámová, K., Elling, L., Křen, V.: Enzymatic preparation of silybin phase II metabolites: sulfation using aryl sulfotransferase from rat liver. *Appl. Microbiol. Biotechnol.* 97, 10391-8 (2013)

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Biotransformation of silybin and its congeners. *Curr. Drug Metab.* 14, 1009-21 (2013)